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13. ABSTRACT *(Maximum 200 words)*

Steroid sex hormones such as estrogen and progesterone affect gingival and osseous tissues and can mediate cytokine production. When administered as oral contraceptives, these hormones affect bone morphogenesis and mediate periodontal disease.[3,4,7,9] Norplant[R], the subdermal contraceptive implant, continually releases a synthetic progestin, levonorgestrel, for five years.[10,11] In order to assess the impact of levonorgestrel on bone cells, murine calvarial cell cultures were harvested, grown to confluence and treated with levonorgestrel, progesterone and estrogen. The majority of the cells grown in these bone cell cultures were phenotypically and morphologically characteristic of an osteoblast. In a group of cultures treated with levonorgestrel, bone cells secreted significantly lower amounts of IL-1β-stimulated IL-6 versus control cultures ($p < 0.05$). Similarly, cultures treated with progesterone secreted significantly lower amounts of IL-1β-stimulated IL-6 versus control cultures ($p < 0.05$), but significantly higher amounts than those treated with levonorgestrel ($p < 0.05$). In a group of cultures treated with the two progestins and estrogen, IL-1β-stimulated IL-6 levels indicated a significant interaction between the hormones ($p < 0.05$). These results suggest that both levonorgestrel and progesterone when used in combination with estrogen may partially inhibit the anti-resorptive effect of estrogen through an increase in IL-6 secretion.

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EFFECT OF LEVONORGESTREL (NORPLANT) ON THE IMMUNE REGULATION OF BONE MORPHOGENESIS IN CALVARIAL CULTURES FROM THE LABORATORY MOUSE (MUS MUSCULARIS)

Arlynn G. Ráez, Carol A. Lapp and David W. Craft

INTRODUCTION

Recent advances in immunobiology have increased the understanding of the mechanisms of host immune responses in periodontal disease. Although bacteria are considered to be the main local factor responsible for periodontal pathogenesis, investigators have identified many different mediators of this process. For example, the role of cytokines in human diseases is a rapidly developing area of study, with interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor (TNF) apparently being the most actively involved in periodontal disease.[1,2] Studies also support the concept that altered levels of steroid sex hormones have a strong influence on the host immune reaction to inflammation.[3]

Steroid sex hormones have direct effects on gingival and osseous tissues and can mediate cytokine production as well. It is well documented that gingival inflammation is exaggerated during puberty and pregnancy, which is a time that represents a marked increase in the hormone levels of women. Other studies have also shown increased gingival inflammation and hyperplasia in women taking oral contraceptives.[3,4,5] Oral tissues are more exposed to the free (unbound) circulating hormone (from saliva and blood) when compared to other tissues (ie. muscle), therefore the oral tissues are more vulnerable to the effects of hormonal change.[3] It has been suggested that there is an immunosuppressive effect from steroid hormones, which results in a decrease in the host's immune response and contributes to the destruction of periodontal tissue.[6]

It has been demonstrated that steroid sex hormones affect inflammation through their actions in the function of polymorphonuclear leukocytes (PMN) chemotaxis[4], as well as by regulation of the circuitry of cytokine action that controls bone remodeling.[2,7] Endogenous sources for the alteration of the total level of circulating sex steroids can be multiple. Decreases in these levels can either be from decreased production (i.e. menopause), increased metabolic breakdown of these hormones, or increased production of carrier proteins. Increased levels on the other hand, can be due to increased production from usual sources and/or tumor sources. The most common reason for excessive circulating levels of sex hormones is the exogenous physiologic or pharmacologic intake of enteric and parenteric contraceptive hormonal devices.[8] The estrogen and progesterone oral contraceptive hormonal effect on bone morphogenesis and periodontal disease has been extensively studied.[3,4,7,9] Norplant[R], the newest contraceptive system, consists of subdermal contraceptive implants which continuously release a synthetic progestin, levonorgestrel, for five years.[10,11] To date, no *in vitro* studies have been reported examining the effects of levonorgestrel on cytokine production in bone morphogenesis. The specific aim of this study is to assay for the presence of IL-1β-stimulated IL-6 in bone cell culture supernatants treated with levonorgestrel and progesterone.

Materials and Methods

Primary Cell Culture

Cells were obtained from fetal murine calvaria. Using an aseptic technique, approximately 160 murine calvaria (*Mus mucularis*-Hsd: (ICR) BR) were collected. These were minced and the fragments obtained were enzymatically digested in 0.05% crude collagenase to dissociate cells from the bone matrix. The calvaria fragment/collagenase mixture was stirred for 20 minutes. The supernate

containing the freed bone cells was washed with 10 ml of Dulbecco's Modified Eagle medium (DMEM) and centrifuged for seven minutes at 1800 rpm. The supernate was discarded and the pellet resuspended with 10 ml Hank's Balanced Salts (HBSS). The mixture was again centrifuged for seven minutes at 1800 rpm. The supernate was discarded and the pellet resuspended in 4.5 ml DMEM. This suspension was then gently layered onto 4.5 ml of Ficoll-Paque. These steps were repeated for four digestions. The four suspensions on Ficoll-Paque were then centrifuged at 1650 rpm for 50 minutes. The buffy coats from the four digestions were combined and washed with HBSS and centrifuged at 1000 rpm for 10 minutes. The supernate was discarded and the pellet resuspended for a second wash in 14 ml of HBSS. Again the supernate was discarded and the pellet resuspended in a final 4 ml volume of DMEM.

Experimental Design

Cells were diluted with DMEM to deliver a concentration of 2 x 10^4 cells per well into 24-well ProNectin F coated plates (Protein polymer Technologies, San Diego, CA). The DMEM used was phenol red-free and contained 10% fetal bovine serum (FBS), non-essential amino acids, 15mM Hepes, Na Pyruvate, Penicillin (100 U/ml), Streptomycin (100μg/ml), Amphotericin B(0.5μg/ml), Gentamicin (10mg/ml) and 1,25 Dihydroxyvitamin D_3 (41.6μl/ml).

Cells were grown to confluence by day 5 at 37°C in 5% CO_2/95% ambient air and monitored for alkaline phosphatase activity throughout the experiment. To start the experiment, the medium was replaced with medium containing either control medium or medium containing levonorgestrel or progesterone on day 5. In addition, half of the samples included 10^{-10}M estrogen. Each steroid was dissolved in ethanol and further diluted in DMEM to a final ethanol concentration below 0.1%.

Control media contained the same ethanol concentration. Following incubation for 24 hours, all samples were stimulated with 0.2 ng/ml of IL-1β. Cultures were harvested on day 7 and growth media supernates were collected, stored at -70°C and assayed for the presence of IL-6. The remaining cell monolayer was solubilized with 0.03N NaOH and 1% sodium dodecyl sulfate and assayed for total protein with the Pierce (BCA) assay.

Steroids

To determine if there was a dose dependent response, the levonorgestrel was added to cell cultures at a final concentration of $5x10^{-9}$M, $5x10^{-8}$M, $5x10^{-7}$ M, $5x10^{-6}$M, which approximated high and low levels found in the plasma of women using NORPLANT^{R}. Progesterone was tested at 5×10^{-8}M. All of the samples were harvested and media changed in the same manner as those samples treated with levonorgestrel.

IL-6 Immunoassay

The IL-6 assay utilized a variation of the quantitative sandwich enzyme immunoassay method consisting of a monoclonal antibody specific for murine IL-6, a biotinylated polyclonal anti-murine antibody, an avidin-horse radish peroxidase conjugate, and substrate (PharMingen, San Diego, CA). Color detection was read at 405nm. A standard curve was generated with known concentrations of recombinant murine IL-6. Concentrations of unknown samples were extrapolated from the standard curve.

Alkaline Phosphatase Assay

The presence of alkaline phosphatase activity was detected using a commercially available test kit, Alkaline Phosphatase, Leukocyte kit (Sigma Diagnostics, St. Louis, MO). Briefly, cover slips of cells were fixed in a citrate-acetone-formaldehyde solution, stained with a diazonium salt solution (sodium nitrate/fast red violet alkaline solution/naphthol AS-BI solution), counter stained with hematoxylin and evaluated microscopically.

Data Analysis

The IL-6 and protein content for each sample were extrapolated from standard curves. Results were described as mg IL-6 per μg protein. These concentrations of treated (with levonorgestrel or progesterone) cell cultures versus non-treated (controls) cell cultures were compared by Kruskal-Wallis analysis of variance and differences among groups were analyzed by Duncan's multiple range test ($p < 0.05$). Cell cultures treated with levonorgestrel and progesterone in combination with estrogen were compared by the multivariant ANOVA ($p < 0.05$).

Results

Although one of the initial goals of this study was to develop a serum-free medium that could be used with the different concentrations of levonorgestrel and progesterone, the use of medium with reduced-serum failed to support osteoblast growth equivalent to that observed with 10% FBS. Therefore, the final DMEM/FBS medium was analyzed for the presence of hormones and growth factors. The levels of estradiol, progesterone, corticosterone, hydroxycorticosterone, insulin growth factor (IGF) binding protein, IGF-I/somatomedin-C, insulin, growth hormone, 17α-hydroxyprogesterone, testosterone, parathyroid hormone, insulin-like growth factor II, prolactin, cholesterol and estrone were found to be below normal physiologic levels of a woman in the follicular

phase of the menstrual cycle.

Protein concentration (μg/well) did not vary significantly among treatment groups ($p < 0.05$). Separate samples were microscopically observed for alkaline phosphatase with all samples indicating activity.

In the group of cultures not treated with estrogen, cells treated with levonorgestrel secreted significantly lower amounts of IL-1β-stimulated IL-6 versus control cells ($p < 0.05$)(Figure 1). Similarly, cultures treated with progesterone secreted significantly lower amounts of IL-1β-stimulated IL-6 versus control cells ($p < 0.05$), but significantly higher amounts than those treated with levonorgestrel ($p < 0.05$).

In the group of cultures treated with progesterone and levonorgestrel in combination with estrogen, there was a significant interaction between the hormones ($p < 0.05$) (Figure 2.). Specifically, at the highest level of levonorgestrel tested (5×10^{-6}M), IL-1β stimulated IL-6 production increased versus the lower concentrations of levonorgestrel ($p < 0.05$). Progesterone treated cells also showed a significant increase in IL-1β stimulated IL-6 production versus the lower levels of levonorgestrel ($p < 0.05$). There was no significant difference between the levonorgestrel (6 x 10-6M) and progesterone.

Discussion

Bone formation is accomplished through the process of cell proliferation, alkaline phosphatase expression, and matrix production mediated in part by osteoblasts. [12,13] These cells in turn regulate osteoclast activity through mediators such as sex hormones and cytokines.[14-16]

Cytokines have been shown to influence bone remodeling and periodontal pathogenesis through regulation of the immune response, hematopoiesis, and inflammation.[17] Interleukin-6 is produced by osteoblasts in response to local bone-resorbing agents, and induces bone resorption by stimulating osteoclastic activity.[18,19] Further, IL-6 mediates the anti-resorptive effect of estrogen, a major regulator and modulator of bone metabolism which has been shown to down-regulate the production of IL-6 by osteoblasts.[7,20-23] Progesterone modulates bone turnover as well as bone formation. Previous studies have shown that progesterone can also reduce IL-6 production by human gingival fibroblasts[24].

The most common source of exogenous steroid sex hormones is the administration of oral contraceptive pills in a combination of an estrogen and a progestin. Norplant contains only a synthetic progestin, levonorgestrel which is molecularly very similar to progesterone. Sustained levels of levonorgestrel produce a strong antiestrogenic effect on the body.[10] Due to the potential changes caused by the continuously elevated levels of this progestin, it was important to determine the effects of levonorgestrel on cytokine production.

Under the conditions of this *in vitro* bone cell model an attempt was made to duplicate the *in vivo* relationship of levonorgestrel with osteoblasts. The murine osteoblasts grown in these calvarial cultures were phenotypically and morphologically characteristic of an osteoblast. In the treatment groups without estrogen there was a significant inhibition of osteoblast secreted IL-6 after treatment with two different progestins. This effect agrees with previous studies in gingival fibroblasts indicating a progesterone mediated decrease in the expression of IL-6 mRNA versus non-treated controls.[24] In addition, levonorgestrel treated cell cultures secreted significantly less IL-6 than those treated with progesterone.

In the treatment groups that included estrogen, there was a combined effect of the estrogen with the two progestins. Interleukin-6 secretion increased for both the progesterone treated cells and the highest concentration of levornorgestrel ($5x10^{-6}M$) treated cells versus non-treated cells. This effect suggests a possible mediation of the individual effect of the three hormones when combined at commonly achieved human physiologic levels.

CONCLUSION

Certainly in the human body there is a complex interaction between hormones, bone morphogenesis and the immune system. These results, if applicable to the *in vivo* osteoblast environment, suggest that both levonorgestrel and progesterone when used in combination with estrogen may partially inhibit the anti-resorptive effect of estrogen through an increase in IL-1β stimulated IL-6 secretion. The implication of this study dictates that future *in vivo* studies be undertaken to examine the clinical and treatment significance of these hormone interactions.

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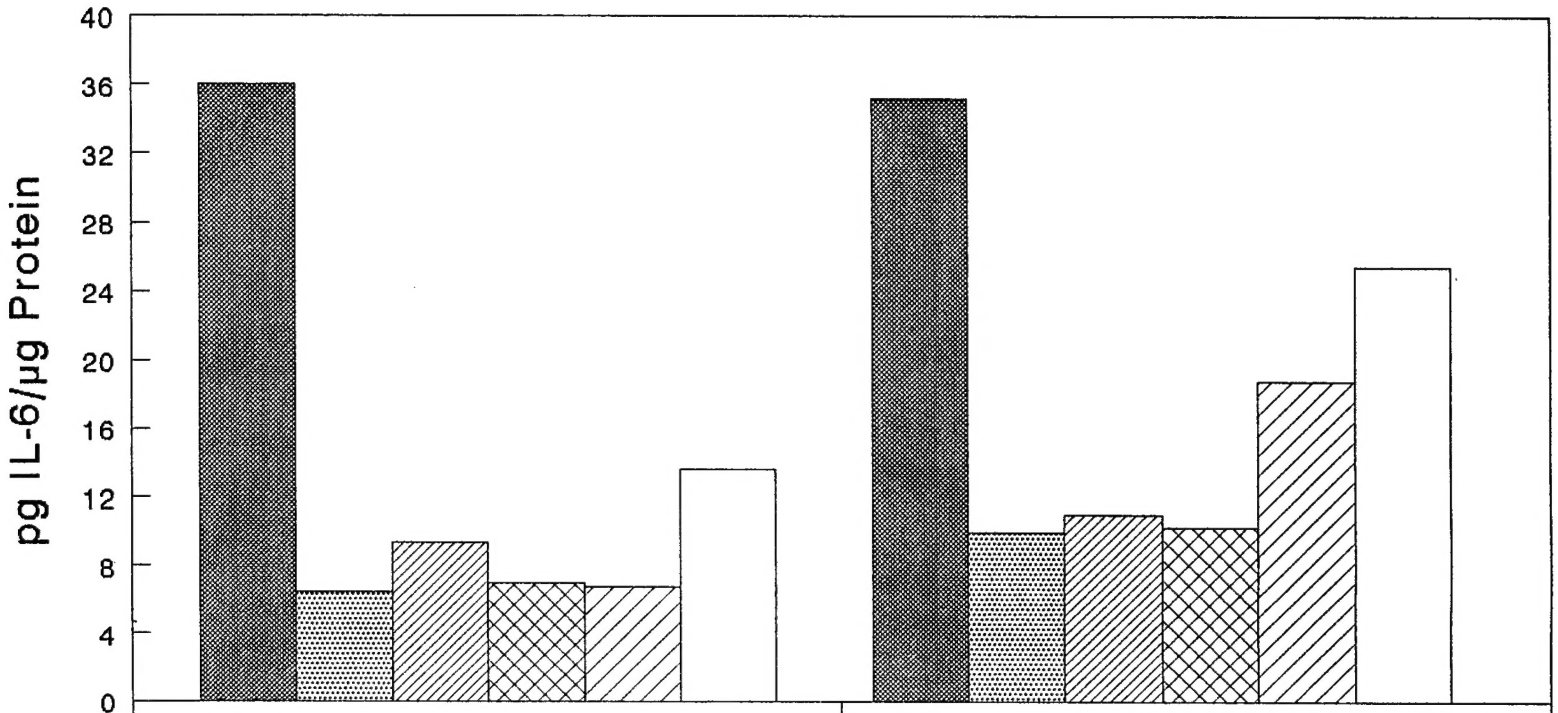
IL-6 Production by Murine Bone Cells



	IL1ß	IL1ß+E2
Control	36	35.2
5x10-9M Levo	6.5	9.95
5x10-8M Levo	9.38	11
5x10-7M Levo	7	10.25
5X10-6M Levo	6.78	18.83
5X10-8M Prog	13.6	25.45

FIGURE 1

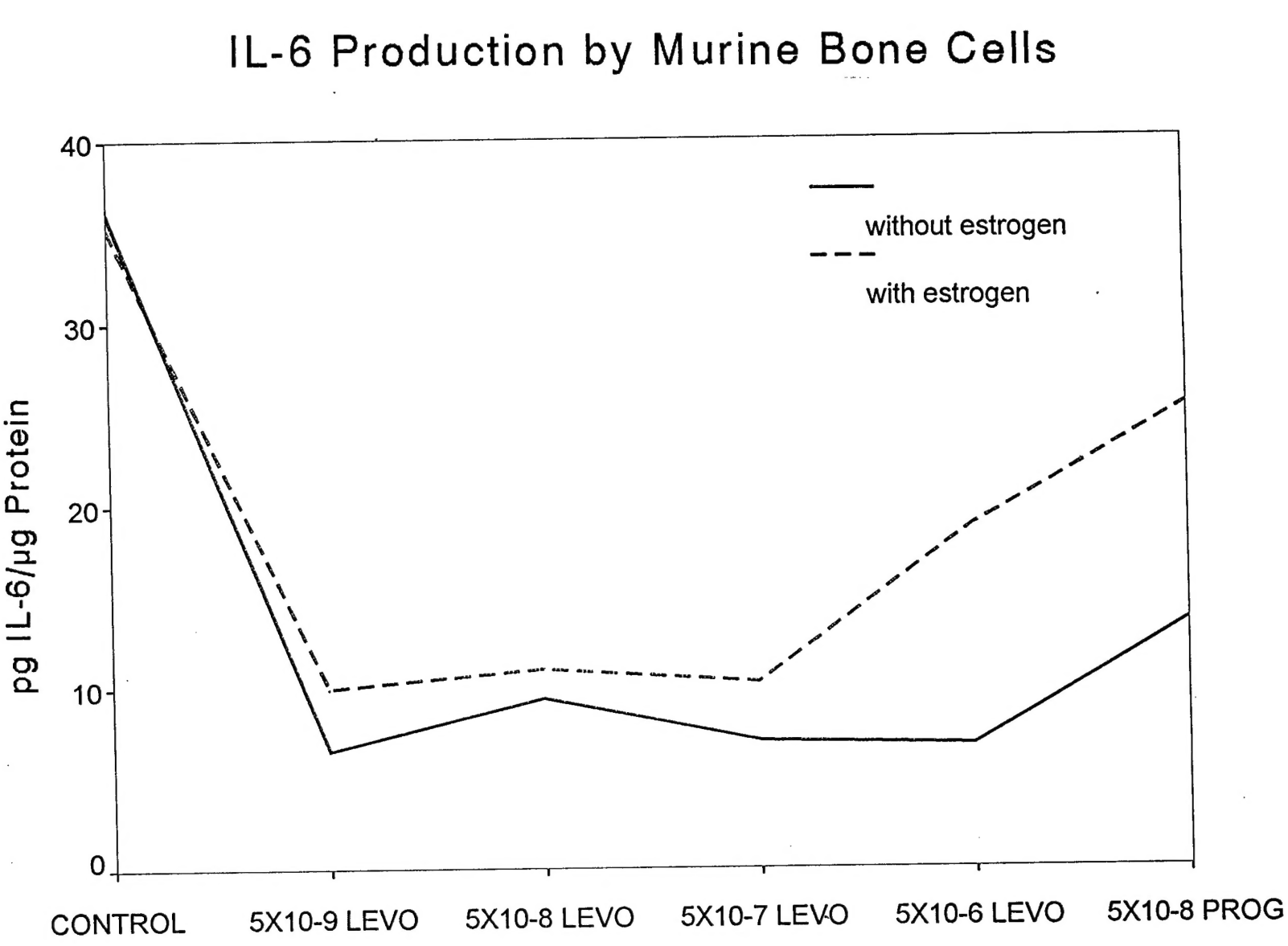

IL-6 Production by Murine Bone Cells
40
30
20
10
0
pg IL-6/µg Protein
without estrogen
with estrogen
CONTROL
5X10-9 LEVO
5X10-8 LEVO
5X10-7 LEVO
5X10-6 LEVO
5X10-8 PROG

FIGURE 2